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ROLE OF MALONDIALDEHYDE-ACETALDEHYDE ADDUCTS IN LIVER INJURY

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Abstract—Malondialdehyde and acetaldehyde react together with proteins in a synergistic manner and form hybrid protein adducts, designated as MAA adducts. MAA-protein adducts are composed of two major products whose structures and mechanism of formation have been elucidated. MAA adduct formation, especially in the liver, has been demonstrated in vivo during ethanol consumption. These protein adducts are capable of inducing a potent immune response, resulting in the generation of antibodies against both MAA epitopes, as well as against epitopes on the carrier protein. Chronic ethanol administration to rats results in significant circulating antibody titers against MAA-adducted proteins, and high anti-MAA titers have been associated with the severity of liver damage in humans with alcoholic liver disease. In vitro exposure of liver endothelial or hepatic stellate cells to MAA adducts induces a proinflammatory and profibrogenic response in these cells. Thus, during excessive ethanol consumption, ethanol oxidation and ethanol-induced oxidative stress result in the formation of acetaldehyde and malondialdehyde, respectively. These aldehydes can react together synergistically with proteins and generate MAA adducts, which are very immunogenic and possess proinflammatory and profibrogenic properties. By virtue of these potentially toxic effects, MAA adducts may play an important role in the pathogenesis of alcoholic liver injury. © 2002 Elsevier Science Inc.

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INTRODUCTION

The chronic consumption of alcoholic beverages is a major cause of liver injury and the development of serious liver disease. Despite considerable research accomplishments in this area of investigation, the biochemical/molecular mechanisms by which ethanol exhibits its hepatotoxic effects remain unclear. Because high levels of reactive aldehydes are generated in the liver during ethanol metabolism, aldehyde-derived modification of proteins (or other macromolecules) has been proposed as a key event leading to liver injury [1–4].

Ethanol oxidation in the hepatocyte results in the formation of acetaldehyde. Furthermore, there is also considerable evidence that chronic ethanol consumption induces oxidative stress and lipid peroxidation in the liver [5,6], resulting in the generation of additional reactive aldehydes, especially malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) [7,8]. All of these aldehydes exhibit reactivity with proteins [9–12], and the presence of acetaldehyde-, MDA-, and HNE- protein adducts have been detected in livers of ethanol-fed rats, micropigs, and humans [2,13,14]. Although these aldehydes alone are capable of adduct formation with proteins, the situation in the liver during ethanol metabolism

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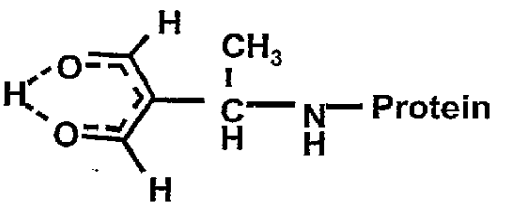
is such that these aldehydes co-exist and may influence each other's reactivity with proteins. In studies exploring this possibility [15], our laboratory has demonstrated that MDA and acetaldehyde react in concert with proteins in a synergistic manner. The presence of both aldehydes strikingly increases each other's binding to proteins and generates hybrid adducts that are clearly different from those adducts formed by either aldehyde alone. These composite MDA-acetaldehyde-protein adducts have been designated as "MAA adducts." MAA adducts are relatively stable products and have been structurally characterized. In addition, MAA adducts appear to induce potent, specific immune responses and possess other unique biological properties relevant to cell injury. Therefore, the focus of this report will be to describe the chemistry of MAA adduct formation and to provide information concerning the functional consequences of MAA adducts as they relate to liver injury.

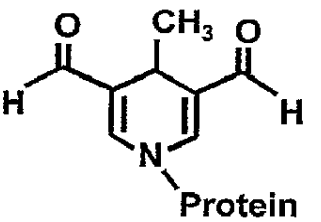
CHEMISTRY OF MAA ADDUCTS

Our initial studies [15] showed that when MDA and acetaldehyde were incubated together with proteins, a marked stimulation of binding of both aldehydes to proteins occurred compared to conditions examining the binding of each aldehyde in the absence of the other. For example, when the model protein, bovine serum albumin (BSA), was incubated in the presence of both MDA and acetaldehyde, MDA caused a marked and concentration-dependent increase in the stable binding of acetaldehyde to BSA. A 13-fold stimulation of binding was observed with equal molar concentrations of the aldehydes, increasing to over 32-fold at a 4-fold molar excess of MDA. Likewise, the presence of acetaldehyde also stimulated the binding of MDA to proteins. This stimulation of aldehyde binding to proteins resulted in a reaction mixture that exhibited highly fluorescent properties. Since these highly fluorescent products were absent when equimolar concentrations of acetaldehyde alone or MDA alone were allowed to react with proteins, it appeared that new and distinct products were generated. These hybrid adducts composed of MDA and acetaldehyde were designated as *MAA* adducts [15].

Upon the basis of chemical analysis [16,17], NMR spectroscopy [17], and immunochemical analysis [16], it appears that two major products result from the reaction of MDA and acetaldehyde with proteins, and these structures are depicted in Fig. 1. One adduct is a highly fluorescent, cyclic product composed of two molecules of MDA and one molecule of acetaldehyde and was identified as the 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde derivative of an amino group (MDHDC adduct). The other adduct (nonfluorescent) is a 1:1 adduct of MDA and acetaldehyde and was identified as the



FAAB Adduct



MDHDC Adduct

Fig. 1. Structures of MAA-protein adducts.

2-formyl-3-(alkylamino)butanal derivative of an amino group (FAAB adduct).

Another important aspect of MAA adduct chemistry is the reaction mechanism that describes the generation of MAA-protein adducts. Our previous studies showed that when MDA and acetaldehyde were allowed to react with proteins, formation of the FAAB (nonfluorescent) adduct preceded the formation of the MDHDC (fluorescent) adduct [16,17]. These findings suggested that the FAAB adduct may serve as a precursor for the generation of the MDHDC adduct. In a recent study [18], it was shown that the first step in the reaction scheme involves the reaction of one molecule of MDA and one of acetaldehyde with an amino group of a protein to generate the FAAB adduct. The other initial step of the overall reaction scheme is the reaction of MDA with an amino group to form a MDA Schiff base (MDA-enamine). After the generation of these two intermediates, the FAAB moiety is transferred to the nitrogen of the MDA-enamine followed by ring closure to produce the MDHDC adduct. It is important to note that the MDHDC product was formed on the nitrogen of the MDA-enamine. For efficient transfer of the FAAB moiety to the

enamine, evidence indicates that these two intermediates likely must be in positions on the protein of close proximity to each other.

IMMUNE RESPONSES TO MAA ADDUCTS

Immunogenicity of MAA adducts

Classical immunization experiments demonstrated that MAA-protein adducts were capable of inducing an antibody response [15]. Further studies showed that MAA adducts could induce an antibody response without the use of adjuvants [19], indicating the potent immunogenicity of these adducts. The antibodies generated were able to recognize and bind either MAA epitopes or epitopes of the unmodified carrier protein [19]. In lower immunizing doses, the antibody response was mainly to the carrier protein; whereas, larger doses induced strong anti-MAA responses. These antibody responses were observed for several carrier proteins that were MAA adducted [19]. Additional studies demonstrated that the antibodies that recognized MAA epitopes were exclusively against the cyclic, fluorescent adduct (i.e., MDHDC adduct) and not against the nonfluorescent (i.e., FAAB) adduct [16].

Initial studies designed to determine the mechanism of the potent immunogenicity of MAA adducts suggested that scavenger receptors, located at the cell surface of peritoneal macrophages or other antigen processing cells, are able to bind and endocytose, process, and present MAA-adducted proteins [19]. This increase in antigen presentation induced by the MAA adduction of proteins would then increase T-helper cell proliferation and activation, ultimately resulting in the enhanced ability of B-cells to produce specific antibodies. Recent support for this mechanism was obtained by investigating the induction of immune responses to MAA adducts in scavenger receptor-A knockout (SRA-KO) mice [20]. These studies showed that the immune response to MAA adducts was attenuated but not eliminated in these knockout mice, suggesting that scavenger receptor-A plays a prominent role, but that other receptors (or other events) may also contribute to generating immune responses to these altered proteins.

Antibodies against MAA adducts in ethanol-fed rats

Chronic ethanol administration to rats has been shown to result in significant circulating antibody titers against MAA-adducted proteins [21]. Competitive inhibition assays demonstrated that antibodies against MAA-modified proteins in the ethanol-fed rats recognized a specific MAA epitope, which was the MDHDC adduct. Additional characterization of the circulating anti-MAA antibodies in the ethanol-fed rats showed that these antibodies were also capable of reacting with MAA-modified proteins from rat liver, including cytosolic, microsomal, and plasma membrane proteins. Further examination of plasma immunoreactivity indicated that ethanol feeding generated antibodies that recognized not only the specific MDHDC epitope on rat liver proteins but also epitopes on the unmodified (native) protein fractions. It also appeared that the magnitude of the increase in the immune response in the ethanol-fed animals was the greatest for the plasma membrane proteins [21]. The mechanism for the increased generation of antibodies to unmodified (native) rat liver proteins in the ethanol-fed rats is unknown, but could be related at least in part to MAA modification of rat liver proteins during long-term ethanol ingestion. As discussed previously, MAA adduction specifically targets proteins for antigen processing and presentation, eventually leading to increased and specific immune responses to both the MDHDC epitope and carrier (native) proteins.

Antibodies against MAA adducts in humans

In a recent study [22], circulating antibodies against MAA adducts were evaluated in patients with alcohol-induced hepatitis or cirrhosis, in patients with non-alcohol-induced liver disease, in heavy drinkers without liver damage, and in healthy controls. The results of this study showed that levels of immunoglobulin G (IgG) that reacted with MAA-modified proteins was significantly elevated in the patients with alcohol-induced hepatitis or cirrhosis compared to subjects in the other three groups. The specific MAA epitope that was recognized by the elevated IgGs was once again the MDHDC adduct. Furthermore, in the alcoholic liver disease group, patients with moderate or severe liver injury displayed significantly higher anti-MAA IgG titers compared with patients with mild liver damage, indicating a correlation of circulating anti-MAA antibody titer to the severity of liver damage.

IN VIVO FORMATION OF MAA ADDUCTS DURING ETHANOL CONSUMPTION

Although the characterization of the chemistry of MAA-protein adducts is important, verification that these adducts are generated during ethanol consumption in vivo represents a key element in evaluating the role of MAA adducts in alcoholic liver injury. In this regard, antibodies against MAA adducts, specifically the MDHDC epitope, as previously discussed, have been detected both in rats [21] and humans [22] chronically consuming alcohol, implying that this adduct formed on

endogenous proteins and stimulated antibody production. Immunoanalysis, employing an affinity purified polyclonal antibody against the MDHDC epitope, as well as a monoclonal antibody to this epitope [16,19], directly confirmed the presence of MAA-modified proteins in livers from ethanol-fed rats (Lieber-DeCarli diet) [15]. Although the presence of the MDHDC adduct in livers from ethanol-fed rats was demonstrated by this study, the formation of the FAAB adduct could not be ascertained because a suitable antibody against the FAAB adduct was unavailable. However, recently our laboratory has obtained indirect evidence that FAAB adducts might also be formed in the liver during ethanol consumption. In this study [18], it was shown that in vitro incubation of liver homogenates, obtained from ethanol-fed rats, with exogenously added MDA markedly increased the levels of MDHDC-protein adducts. Because the FAAB adduct has been shown to be a precursor in the formation of the MDHDC adduct upon reaction with a MDA-enamine [18], these findings would strongly suggest the presence of a pool of FAAB-protein adducts in the livers of ethanol-fed animals. Furthermore, these results strongly suggest that MDHDC-protein adduct formation in vivo may very well proceed by the same reaction scheme that describes in vitro formation of this adduct.

Worrall and coworkers have also demonstrated the formation of MAA adducts in rats fed the Lieber-DeCarli ethanol containing diet. MAA adducts were detected in the livers as early as 2 weeks of feeding ethanol and reached maximum levels after 6 weeks [23]. MAA adduct formation with plasma proteins was shown after 6 weeks of ethanol administration, although the degree of modification was less than that observed for liver proteins [23]. In addition, in the same study circulating antibodies against MAA proteins in the ethanol-fed rat were also identified. In a subsequent study, Worrall et al. [24] were able to demonstrate the presence of MAA adducts in cardiac muscle obtained from ethanol-fed rats. It appears that in all cases referred to above that the major MAA adduct detected in these studies was again the MDHDC adduct.

In summary, several studies have demonstrated the formation of MAA adducts, especially in the liver, during ethanol consumption. Thus, it appears that in addition to acetaldehyde, MDA, and HNE adducts, MAA-adducts represent another important aldehyde-derived modification of proteins elicited by in vivo ethanol metabolism. In fact, it could be argued that because acetaldehyde and MDA can bind in a synergistic manner to proteins, conditions in the liver during ethanol oxidation would favor MAA adduct formation over the formation of the individual aldehyde adducts; and therefore, MAA adducts may very well represent the major aldehydic adduct.

FUNCTIONAL CONSEQUENCES OF MAA ADDUCTS AND THEIR ROLE IN LIVER INJURY

An important aspect regarding the relevance and significance of MAA adducts, in addition to demonstrating their formation during ethanol consumption, is the biological effects of these adducts, especially as they relate to liver injury and to cell injury in other organs. Recent studies have described some interesting and unique biological properties of MAA adducts which may be relevant to their role in inducing liver damage, and the findings from these studies will be summarized in this section.

Influence of MAA adducts on the immune system as it relates to liver injury

Many of the clinical features of alcoholic liver disease suggest that immune effector mechanisms may be contributing to liver tissue damage, and many earlier studies have implicated immune factors in the pathogenesis of alcoholic liver disease [25]. As previously discussed in an earlier section of this report and reported in detail in a recent article summarizing the proceeding of a recent workshop at the 2000 ISBRA Meeting in Yokohama, Japan [20], MAA adducts induce a potent immune response, which may represent an important immunotoxic event relevant to the pathogenesis of liver injury. The following experimental findings are consistent with this hypothesis: (i) MAA adducts induce antibody responses against MAA epitopes as well as to epitopes on the carrier protein including "self" epitopes in the case of MAA adducted liver and plasma proteins; (ii) These immune responses occur in the absence of adjuvant, are rapid in onset, and appear to be restricted to the production of the IgG1 isotype; (iii) Circulating antibodies against MAA adducts have been observed in ethanol-fed animals and in humans with alcoholic liver disease. In the latter case, antibody titers correlated with the severity of liver injury. In addition, the presence of circulating antibodies to "self" proteins (perhaps generated as a result of MAA adduction) has also been reported in ethanol consuming animals; (iv) MAA modification of the hepatitis B antigen (in the absence of adjuvant) enhanced the induction of a cytotoxic T-cell response.

Overall, these findings suggest that MAA adducts could contribute to the development of immunotoxic reactions that could induce alcoholic liver injury by stimulating the production of antibodies and/or T-cells against MAA epitopes (neoantigens) and/or autoantigens (self-epitopes). Therefore, the MAA adduction of proteins could be a mechanism by which harmful immunogens are generated and trigger destructive immune responses targeting liver cells.

Effects of MAA adducts on hepatic endothelial and stellate cells

The in vitro incubation of isolated liver endothelial cells (LECs) with MAA adducts has been shown to stimulate the secretion of several cytokines and chemokines, including tumor necrosis factor-α, monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-2 (MIP-2) [20]. Additionally, the exposure of LECs to MAA adducts upregulated the expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), L-selectin, and P-selectin [20]. MAA adducts also stimulated the secretion of fibronectin by LECs [20]. This latter finding is important because fibronectin has been implicated in the activation of hepatic stellate cells (HSCs), the principal cell type in the liver responsible for the increased deposition of extracellular matrix proteins that characterizes fibrosis and cirrhosis [26].

In addition to activating LECs, MAA adducts also directly affect the function of HSCs. Exposure of isolated HSCs to MAA adducts resulted in a dose- and time-dependent increase in the secretion of the two chemokines, MCP-1 and MIP-2, and in the upregulation of ICAM [27]. Further studies showed that MAA adducts also increase the secretion of urokinase-type plasminogen activator (uPA) via a protein kinase C- (PKC) dependent pathway [28].

These MAA-induced effects on LECs and HSCs appear to be mediated by MAA adducts binding to surface receptors on these cells and initiating signaling cascades [20,28]. Although the specific receptors mediating these effects have not been identified as yet, preliminary studies indicate that they may be members of the scavenger receptor family [20]. Overall, these studies strongly suggest that the activation of LECs and HSCs by MAA-modified proteins may be one mechanism by which proinflammatory and profibrogenic processes are initiated and may play a significant role in the development and/or progression of liver injury.

PERSPECTIVES AND SUMMARY

MAA-protein adducts appear to be a major aldehydic adduct that forms in the liver and other organs during chronic alcohol consumption. These adducts induce a potent immunogenic response and possess proinflammatory and profibrogenic properties. Therefore, upon the basis of what is known about the chemistry of MAA adducts and their biological actions, the following series of events describing the role of MAA adducts in the pathogenesis of alcoholic liver injury is proposed: in the early stages of ethanol consumption, nonfluorescent MAA adduct (i.e., FAAB adduct) formation would be favored because acetaldehyde concentrations would likely exceed those of MDA. This situation would result in a pool of FAAB adducts in the liver. With more prolonged ethanol intake, oxidative stress and lipid peroxidation would occur, resulting in elevated MDA levels, which would be available to generate Schiff base adducts (MDA-enamines) with protein amino groups. The Schiff bases would be available to accept the transfer of the FAAB moiety from the already preformed pool of these nonfluorescent adducts, forming the fluorescent MAA adduct (i.e., MDHDC adduct). This adduct, which has been shown to be very immunogenic and to possess proinflammatory and profibrogenic properties, could by virtue of these potentially toxic effects contribute to the pathogenesis of alcoholic liver injury.

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ABBREVIATIONS

BSA—bovine serum albumin
FAAB—2-formyl-3-(alkylamino)butanal
HNE—4-hydroxyl-2-nonenal
HSCs—hepatic stellate cells
ICAM-1—intercellular adhesion molecule-1
IgG—immunoglobulin G
LECs—liver endothelial cells
MAA—malondialdehyde-acetaldehyde
MDA—malondialdehyde
MDHDC—4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde
MCP-1—macrophage chemotactic protein-1
MIP-2—macrophage inflammatory protein-2
PKC—protein kinase C
uPA—urokinase-type plasminogen activator